(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

09/997423

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 April 2002 (18.04.2002)

PCT

(10) International Publication Number
WO 02/31209 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/US01/31932

(22) International Filing Date: 12 October 2001 (12.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/240,585 13 October 2000 (13.10.2000) US

(71) Applicant *(for all designated States except US)*: **THE GOVERNMENT OF UNITED STATES OF AMERICA, as represented by the SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES, THE NATIONAL INSTITUTES OF HEALTH** [US/US]; Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US).

(72) Inventors; and

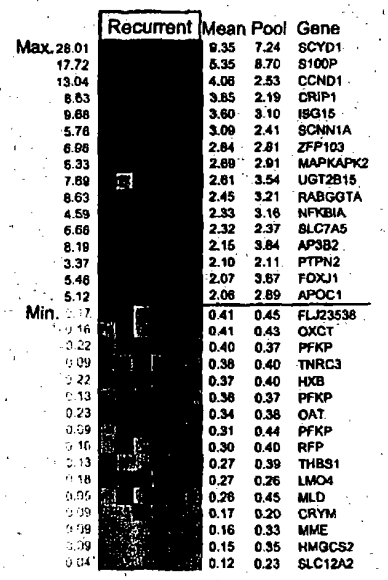
(75) Inventors/Applicants *(for US only)*: **MOUSSES, Spyro** [CA/US]; 257 Congressional Lane, Apt. 417, Rockville, MD 20852 (US). **KALLIONIEMI, Olli, P.** [FI/US]; 1083 Grand Oak Way, Rockville, MD 20852 (US). **BUBENDORF, Lukas** [CH/CH]; Drosselstrasse 31, CH-4106 Therwil (CH).

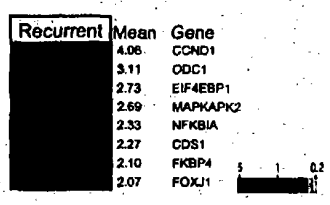
(74) Agent: **HARDING, Tanya, M.**; Klarquist, Sparkman, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).

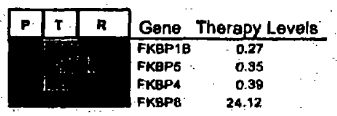
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,

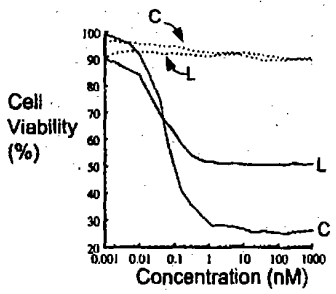
[Continued on next page]

(54) Title: GENES RELATED TO DEVELOPMENT OF REFRACTORY PROSTATE CANCER









(57) Abstract: The present disclosure provides hormone-refractory prostate cancer (HPRC)-related nucleic acid molecules and proteins useful for the detection of neoplasms, particularly prostate and more specifically hormone-refractory prostate cancers. Also provided are methods of using these biological materials in the diagnosis, staging, detection, and treatment of neoplasia, and particularly hormone-refractory prostate cancer.

WO 02/31209 A2

LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

methods. Specific arrays are contemplated that are constructed using molecules identified at such different confidence levels.

In particular, the techniques disclosed herein have uncovered many genes not previously associated with prostate cancer progression, and particularly not previously associated with HRPC. These newly correlated genes include those represented by the following Image ID Clones: 781047, 785778, 842968, 769921, 898286, 204214, 814701, 435076, 531319, 415089, 898062, 453107, 785707, 795936, 700792, 34778, 46182, 769921, 783697, 451907, 711768, 416833, 810711, 789204, 789182, 725454, 951142, 49352, 273546, 46717, 855487, 41117, 26578, 684655, 45233, 814117, 810552, 739511, 283315, and 897774.

Of the 59 androgen-dependent sequences whose expression decreased most after castration (labeled "Decreasing" in Table 2), 58 (98.3%) displayed restored transcript levels in the recurrent tumors, indicating re-activation of androgen-dependent genes in the absence of a ligand.

Tissue microarrays consisting of 50 xenografts and 440 clinical specimens from all stages of prostate cancer progression were utilized to validate potential drug target genes using mRNA *in situ* hybridization and protein immunohistochemistry. Measured by cDNA microarrays, S100P (encoding a calcium-binding protein) was among the most highly overexpressed genes in the CWR22R recurrent tumors; this gene was also highly expressed in the majority of hormone-refractory clinical prostate cancers, but rarely (<10%) in benign prostate lesions.

The temporal gene expression changes identified herein facilitate identification of candidate drugs for hormone-refractory prostate cancer. FKBP5 for example was identified and its utility as a therapeutic target was validated using tissue microarray analysis (see Example 3). Based on such leads, Rapamycin, MS-275, and TSA were tested for their effectiveness in influencing prostate cancer cell growth. These drugs target some of the candidate genes described herein. As described in Example 4, the inventors found that these drugs inhibit CWR22R prostate cancer cell growth *in vitro*. Thus, incorporating cDNA microarray technologies for genomics-based discovery of therapy response genes, with high throughput tissue microarray analysis, provides a new paradigm to identify, prioritize, and validate novel diagnostic and drug targets, as herein described for hormone-refractory prostate cancer.

The identified HRPC-related genes represent putative mediators of hormone therapy response and resistance, and as such are candidate targets for the development of novel therapeutics to maintain prostate cancer in regression following hormone ablation therapy. The utility of these genes as candidate drug targets and biomarkers is demonstrated herein by first using tissue microarrays for high throughput translation to clinical samples, and then selecting drugs that might target these genes. Analysis of cDNA microarray data with template based gene clustering and high throughput translation using tissue microarrays introduces a new, generally applicable paradigm for applying functional genomics to identify genetic programs that mediate a responses to a variety of *in vivo* therapies.

It is contemplated that certain of the HRPC-related genes identified herein encode or correspond to soluble proteins, while other encode or correspond to membrane associated or membrane integral proteins, some of which are exposed at least to a certain extent on the exterior of a cell in which they are expressed. In some embodiments, those HRPC-related molecules that are expressed at or on the surface of a cell are selected as therapeutic targets, for instance for targeting with an antibody-based therapy, which is facilitated by the access of the HRPC-related molecule to the extracellular matrix. These HRPC-related molecules may be described as being "drug accessible."

The disclosure is further illustrated by the following non-limiting Examples.

EXAMPLE 1

Identification of Genes with Altered Expression in Hormone Refractory Prostate Cancer

This example provides a description of how the disclosed HRPC-related nucleic acid molecules were identified. These HRPC-related nucleic acid molecules show differences in expression during prostate cancer development, and particularly during hormone ablation therapy and subsequent progression to a hormone-refractory condition.

Methods and Material:

Xenografts and Cell Line: CWR22 is a serially transplantable, prostate cancer xenograft that was derived from a Gleason score 9 primary human prostate cancer with osseous metastasis (Wainstein *et al.*, *Cancer Res.* 54:6049-6052, 1994). CWR22 is highly responsive to androgen deprivation, with marked tumor regression after castration (Cheng *et al.*, *J. Natl. Cancer Inst.* 88:607-611, 1996; Nagabhushan *et al.*, *Cancer Res.* 56:3042-3046, 1996; Myers *et al.*, *J Urol.* 161:945-949, 1999). About half of the treated animals develop recurrent tumors (CWR22R) over a time frame of from a few weeks to several months. CWR22R is not dependent on androgen and is able to grow in castrated animals.

Thirteen fresh-frozen human prostate xenograft tissues were recovered from mice at different stages of hormonal therapy (four primary untreated CWR22, CWR22 after 0.5 days, 2 days, 4 days, 8 days and 16 days after castration, and four independent hormone-refractory CWR22R strains). LNCap (ATCC) and CWR22R (established from recurrent CWR22R xenografts) cell lines were cultured in RPMI1640 (BibcoBRL) with 10% Fetal Bovine Serum (GibcoBRL) at 37 °C and 5% CO_2. The tumors were flash frozen and stored at -70 °C. RNA was extracted by crushing the tumors in liquid nitrogen and used directly for mRNA isolation with the FastTrack 2.0 Kit (Invitrogen Corp., Carlsbad, CA).

cDNA Microarrays: The cDNA microarrays consisted of 6605 elements representing different (non-redundant) genes. PCR products from sequence-verified clones (Research Genetics, Huntsville AB) were prepared and printed at high density onto glass slides according to previously described protocols (Mousses *et al.*, "Gene Expression Analysis by cDNA Microarrays," in

(ODC1), lactate dehydrogenase A (LDHA), a disintegrin and metalloproteinase domain (ADAM9), v-fos FBJ murine osteosarcoma viral (FOS), and andromedulin (ADM).

The template based, supervised cluster of 59 genes (filtered for greater than three-fold change; greater than 0.8 maximum correlation coefficient; only decreasing templates) (listed in Table 2, and labeled "Decreasing"), representing the genes with the largest decrease after castration, had extensive overlap (51 of 59 genes in common, Image Clone ID numbers 767817, 840567, 785778, 283315, 814117, 45233, 29063, 684655, 134719, 24145, 898062, 855487, 42059, 53316, 273546, 293727, 49352, 725454, 856427, 789182, 531319, 149013, 47833, 626716, 789204, 453107, 416833, 711768, 451907, 66406, 129865, 796646, 204214, 898286, 814701, 416833, 43550, 435076, 207358, 769921, 204257, 207288, 811015, 700792, 131316, 795936, 126650, 781047, 774446, 785707, 415089) with the hierarchical (unsupervised) cluster of 139 genes (Table 5). However, the unsupervised cluster was not inclusive of all the genes that responded to the therapy (since it only contained 139 of the 305 genes with a profile that fit a decreasing template with a minimum two-fold difference and >0.7 max. correlation coefficient). Furthermore, although supervised clustering did identify that at least 74 genes increased by more than three-fold and that fit an increasing template with more than 0.8 correlation coefficient, it was difficult to identify a coherent unsupervised cluster of increasing genes.

By template based clustering and filtering the data, a temporal gene expression program (fingerprint), or cluster of genes, was identified that had the largest expression decrease after castration and the best correlation to a decreasing temporal template (FIG 2). The genes are plotted from early repressed genes on the top, and gradually being repressed at later time points down the list to the bottom genes that had a late onset repression. Investigation of the genes in this list of 59 revealed at least eight genes previously known to be stimulated by androgens, and probably direct targets of the AR. The identification of these AR responsive genes in this cluster further substantiates the utility of template based gene clustering in identifying therapy response associated genes and suggests that other genes in this list may be previously unknown AR responsive genes.

Further examination of the genes in this cluster revealed that it is very rich in several important cell cycle regulators. These include genes known to be associated with cell growth of prostate cancer including PCNA, ornithine decarboxylase 1, c-fos, and tubulin. Most of the genes in this cluster however, are novel cell cycle regulators that were not previously associated with androgen ablation in prostate cancer. These include the following (Image ID Clone numbers in parentheses):

two **BUB** (budding uninhibited by benzimidazoles) genes, which regulate the cell cycle at the mitotic checkpoint by controlling chromosome segregation and responding to spindle disruption (781047, 785778, 842968);

UBCH10, a cyclin-selective ubiquitin carrier that regulates the destruction of mitotic cyclins (769921);

CDKN3, a CDK-2 associated dual phosphatase (700792);

CDC2 delta T which regulates entry into S-phase and mitosis (898286);

CDC18L, which initiates replication (204214);

CKS2, a kinase that activates CDC28 (725454);

MAD2L1, which regulates mitotic checkpoints especially sensitive to kinetochore and spindle loss (814701);

CENPF, a centromere/kinetichore cell cycle protein (435076);

STK12, a chromosome associated kinase that plays an important role in centrosome duplication regulation, aneuploidy, and amplification (531319);

NEK2 a protein kinase that regulates G2-M transition (415089);

CDC20, responsible for nuclear movement prior to anaphase and chromosome separation (898062); and

CDC45L, required for the initiation of DNA replication (453107).

The abundance of growth regulatory genes in this cluster, and of genes known to be direct targets of the androgen receptor, provides further supports that genes in this cluster are dependent on androgens and are mediating the AR dependent growth arrest following androgen ablation.

Transcript levels of the genes in this cluster are restored when therapy fails, suggesting that these are also the genes that mediate the androgen-independent growth in recurrent tumors. These observations are also consistent with the hypothesis that resistance to therapy occurs through an androgen independent activation of the AR.

In addition, several genes were repressed that have never previously been associated with cellular proliferation. FKBP5, for example, was repressed by as much as 5.8-fold after castration. This may be a direct effect of decreased androgen receptor transcriptional activation. FKBP5 has been associated with the glucocorticoid receptor, and targeting of FKBP proteins has been shown to lead to deregulation of several signal transduction pathways.

Another gene that showed a large amplitude change after castration, with unknown consequence, is transmembrane 4 superfamily member 1 (7.1 fold decrease). Conversely, transmembrane 4 superfamily member 3 increased after castration (3.2 fold). Putative signaling molecule serine/threonine kinase 12 (7.0 fold decrease) and insulin induced gene 1 (8.1 fold decrease) also showed substantial expression level changes after castration. Like the known cell cycle regulators, the expression of all these other genes is restored in the recurrent tumors. It is likely that these genes mediate growth arrest after therapy, and tumor re-growth after development of therapy resistance, and therefore these genes are ideal drug target candidates.

In addition, some important genes that changed but did not make the top 59 list include S100P, ID3, PSA and c-myc mRNA, which decreased by 5.2, 2.85, 2.77 and 3.01 fold respectfully during regression (FIG 2). These were not included in the primary list either because they did not meet the 3 fold cut-off, or because the maximum correlation coefficients were less than 0.8 (0.51

0.67, 0.71 and 0.50). Of this group, S100P and ID3 are especially good candidate drug targets because they are also over-expressed in recurrent CWR22R relative to their primary counterparts (Table 2).

Most, but not all, of the genes that show increased expression following therapy response generally remained elevated in the recurrent tumors. This is contrary to the repressed genes, whose transcript levels were largely restored. Some genes, however, increased during therapy and then were restored in the recurrents. This group includes: the UDP glycosyltransferase 2 family, polypeptide B15 and UGT2B4, sialyltransferase 1 (beta-galactoside alpha-2,6-sialytransferase), fatty-acid-Coenzyme A ligase, human metallothionein (MT)I-F gene, tumor suppressor PTEN, cadherin 3, placental-cadherin, gelsolin (amyloidosis, Finnish type), TAP binding protein (tapasin), and several other transcripts. The increase in PTEN indicates that the AKT S6 kinase pathway may be inhibited following castration, suggesting that therapeutic intervention with rapamycin may mimic this inhibition in recurrent tumors.

EXAMPLE 2

Identification of Further Genes with Altered Expression in Hormone Refractory Prostate Cancer

Using different microarrays, and methods essentially similar to those described above in Example 1, additional HRPC-related nucleic acid molecules were identified and further characterized. These HRPC-related nucleic acid molecules also show differences in expression during prostate cancer development, and particularly during hormone ablation therapy and subsequent progression to a hormone-refractory condition.

Methods and Material:

Methods and materials were essentially as described in Example 1, except that additional custom cDNA microarrays were used, constituting 6605 to 8000 elements (sequence verified clones from Research Genetics, Huntsville, Alabama), representing different (non-redundant) transcripts including 4032 to 7700 known (named) genes (Mousses *et al.*, *Funtional Genomics: Gene Expression Analysis by cDNA Microarrays* Livesey and Hunt (eds). Oxford University Press: Oxford, pp. 113-137, 2000.). All xenografts were analyzed at least twice. Either LNCap or CWR22R were used as a reference and labeled with Cy5. The reference cDNA was simultaneously hybridized with Cy3 labeled test specimens on a cDNA microarray as previously described (Mousses *et al.*, *Funtional Genomics: Gene Expression Analysis by cDNA Microarrays* Livesey FJ and Hunt SP (eds). Oxford University Press: Oxford, pp. 113-137, 2000.). Fabrication of the microarray slides, image generation, and the software used for the ratio analysis, and bioinformatics were as described above. Mousses *et al.*, *Funtional Genomics: Gene Expression Analysis by cDNA Microarrays* Livesey and Hunt (eds). Oxford University Press: Oxford, pp. 113-137, 2000.

Template-based clustering was performed as described above.

Most Systematically Altered Genes

Another set of genes was identified that show d differential expression between primary and recurrent tumors. Based on the mean gene expression ratios from six recurrent and four primary tumors, expression levels of 104 of the 3495 informative genes (3.0%) were significantly (2-fold or more) increased, and those of 60 genes (1.7%) decreased in the recurrent tumors. FIG 3A shows 30 genes (out of a total of 164 differentially expressed genes) that were most systematically altered in the recurrent tumors. These genes include SCYD1, S100P, CCND1, CRIP1, ISG15, SCNN1A, ZFP103, MAPKAPK2, UTG2B15, RABGGTA, NFKBIA, SLCYA5, AP3B2, PTPN2, FOXJ1, and APOC1 (all upregulated) and FLJ23538, OXCT, PFKP, TNRC3, HXB, PFKP, OAT, PFKP, RFP, THBS1, LMO4, MLD, CRYM, MME, HMGCS2, and SLC12A2 (all downregulated).

Among the 164 genes were several genes coding for proteins that either converged on the PI3K/AKT/FRAP pathway or represented direct targets of macrolide drugs (such as rapamycin and FK506). As highlighted in FIG 3B, several genes that were androgen-responsive and re-expressed in the recurrent tumors (CCND1, ODC1, EIF1EBP1, MAPKAPK2, NFKBIA, CDS1, FKBP4, and FOXJ1) met these criteria and suggested involvement of rapamycin-sensitive signaling in hormone-refractory tumors.

These findings appeared to indicate that rapamycin-sensitive gene products and signaling pathways play a role in androgen independent growth in the recurrent tumors. To further evaluate this hypothesis, the effects of rapamycin and FK506 on the growth and viability of a cell line established from the recurrent CWR22R xenografts were studied. Rapamycin is a known inhibitor of the PI_3K/AKT/FRAP pathway (Kunz *et al.*, *Cell*, 73:585-596, 1993; Brunn *et al.*, *EMBO J.*, 15:5256-5267, 1996; Sekulic *et al.*, *Cancer Res.*, 60:3504-3513, 2000), and FK506 targets many of the same intracellular proteins as rapamycin. Death of the hormone-independent CWR22R cells was observed at very low doses of rapamycin (IC50 ~ 0.1 nM) (FIG 3D), whereas hormone-responsive LNCap prostate cancer cell lines exhibited partial inhibition, even at high doses (FIG 3D). FK506 treatment did not have an inhibitory effect on either the CWR22R or LNCap cells even at the highest doses tested (greater than 80% cell survival at a dose of 10 mM). The results are based on two different cell lines that are not isogenic and may have other differences contributing to the observed effects. However, both these results of the global-scale gene expression studies and the data from the in vitro sensitivity testing, indicate that further studies are warranted to explore rapamycin as a candidate drug for the treatment of hormone refractory prostate cancers.

Cancer cells exhibit greater than a 1000-fold (IC_{50} ranging from <1 nM to >10 mM) variability in their sensitivity to rapamycin, possibly reflecting mechanisms of intrinsic resistance (Hosoi *et al.*, *Mol. Pharmacol.*, 54:815-824, 1998). Cancer cells that have activated genes and pathways that signal through the PI3K/AKT/FRAP pathway may be particularly sensitive. For example, IGF-1 receptor activation is associated with the efficacy of rapamycin treatment in childhood sarcomas (Dilling *et al.*, *Cancer Res.* 54:903-907, 1994). Several growth factors and related genes that we observed to be overexpressed in the recurrent prostate cancers relative to the

primary tumors (such as HGF, VEGFC, FGF2, IGFBP3, PDGFA, LTBP4, GFR, PGF, ITPKB, CDS1, and FKHL13) could have similarly contributed to the activation of the PI3K/AKT/FRAP pathway and alterations in the rapamycin target expression.

Finally, the two macrolide drugs rapamycin and FK506 bind similar intracellular targets but have different biological effects in hormone-refractory prostate cancer. These differences may be informative in elucidating those molecular pathways that are most critical for progression of prostate cancer. Rapamycin and FK506 both bind to FKBP12 (FK506-binding protein 12) (Sabers *et al.*, *J. Biol. Chem.*, 270:815-822, 1995; Liu *et al.*, *Cell* 66:807-815, 1991). Rapamycin-FKBP12, but not the FK-506-FKBP12 complex, inhibits FRAP (FKBP-Rapamycin Associated Protein), a member of the phosphoinositide-3-kinase related kinases that regulate translation following mitogenic activation of the PI3K/AKT/FRAP pathway. In contrast, FK506, but not rapamycin, inhibits calcineurin activity (Liu *et al.*, *Cell* 66:807-815, 1991). This suggests that, of the many known and unknown targets of rapamycin and FK506, FRAP and the activity of the PI3K/AKT pathway is a more likely candidate than calcineurin as a drug target in hormone-refractory prostate cancer.

This example clearly illustrates that transcriptional profiling can be used to identify candidate drugs for treatment of prostate cancer, and this approach generally, as well as the present findings more specifically, can be used for a basis of such treatment decisions.

EXAMPLE 3

Analysis of Specific Genes

A direct comparison of a pool of four primary CWR22 xenografts and four recurrent CWR22R xenografts was done by labeling one pool with Cy5 and the other with Cy3 and hybridizing them together (Direct P/R column in Table 2). This resulted in 251 genes (3.8% of the 6605 genes assayed; listed in Table 6) that were differentially expressed at the 99 % confidence level. This analysis was also done against the standard reference for each tumor individually and in pools with the most consistently differentially expressed genes shown in Table 4.

One of the most highly differentially expressed genes is a calcium binding protein, S100P. It was found to be expressed 16 times (by cDNA microarray analysis) to 100 times (by Northern hybridization analysis) higher in one recurrent xenograft compared to the primary. The S100P protein has been reported to be associated with increased survival and loss of senescence in breast cancer cells. This data indicates that S100P expression may be androgen dependent, as would be expected if it is involved in prostate cancer progression.

Several immunophilin-like proteins were also identified as being differentially expressed. FKBP5, in addition to being overexpressed by about two-fold on average, is one of the most repressed genes after castration. During recurrence, its expression is restored to higher levels than in the primary. FKBP5 is a member of the large immunophilin chaperone proteins, which have been shown to interact with HSP90 and several steroid receptors. The expression of this protein not only appears to be regulated by the androgen receptor function, but also may affect androgen receptor

activity by protein folding of the nascent receptor or by modulating its binding affinity to ligands. There are several inhibitors (*e.g.*, FK506 and rapamycin) that bind to immunophilins, resulting in either calcineurin inactivation and or the inhibition the phosphotidylinositol 3-kinase/PTEN/AKT/FRAP pathway (Zhong *et al.*, *Cancer Res.* 60:1541-1545, 2000).

The phosphorylated substrates of this pathway include calcineurin and ties into the calcium signaling pathway. In addition, IkB (which regulates NFkB), NFAT, and BAD are each substrates for this pathway and are all involved in regulation of cell survival. Decreased expression of PTEN, and increased expression of CDP-DG synthase, IkB, PHYH, and several other changes also converge on, and possibly alter activity of, this pathway. Drugs that target immunophilins such as FK506 and rapamycin have been shown to inhibit this pathway at the level of FRAP, leading to (1) loss of activity for kinases with mitogen activated protein kinase (MAPK) like substrates and (2) inactivation of calcineurin. Differential gene expression data disclosed herein indicate that such drugs such as rapamycin and FK506 could have a dual role in preventing androgen independent progression of prostate cancer, by both (1) blocking signal transduction from the phosphotidylinositol 3-kinase/PTEN/AKT/FRAP pathway and (2) interfering with androgen receptor protein folding and assembly. This is an example of the differential gene expression discussed herein, to assist in selecting new therapies for treatment of primary and recurrent (hormone-refractory) prostate cancer.

EXAMPLE 4

Tissue Microarray Analysis of Candidate Biomarkers

This example provides in-depth analysis of several HRPC-related genes, including illustrations of the clinical relevance of these genes in prostate cancer progression and staging. High throughput molecular validation of candidate genes in clinical specimens was accomplished by using tissue microarray technology to assess the utility of these HRPC-related genes as biomarkers and drug targets. Using a tissue microarray in this fashion represents an important method to cross-validate data from experimental systems and human cancer specimens.

Tissue microarray methods were carried out essentially as described above, and as known in the art; see, for instance, Kononen *et al.*, *Nat Med.* 4(7):844-847, 1998) Clinical translation of novel gene products where an antibody does not exist can be detected on tissue microarrays using isotopic in situ hybridization (ISH) (Kononen *et al. Nat Med.* 4(7):844-847, 1998; Frantz *et al.*, *J Pathol.* 195(1):87-96, 2001)

S100P

The prevalence of S100P protein overexpression was investigated by immunohistochemistry, in 440 human prostate cancer specimens at various stages of progression. These specimens were arrayed in a prostate cancer progression tissue microarray (Bubendorf *et al.*, *J Natl Cancer Inst* 91:1758-1764, 1999 and Bubendorf *et al.*, *Cancer Res.* 59:803-806, 1999). This

array also contained about 50 different prostate cancer xenograft samples, including those used in the cDNA microarray experiments.

S100P mRNA was measured by three different methods in nine xenografts. cDNA microarray ratios measure the expression of S100P transcript by the amount of cDNA hybridized relative to the standard reference. Northern analysis with a PCR amplified fragment of the S100P against a blot of the same RNA used in the cDNA microarray analysis produced a fragment of expected size (~0.5 kb). Northern hybridization bands were quantified using ImageQuant software from a scanned autoradiogram. An mRNA *in situ* hybridization (ISH) was performed by radio-labeling eight non-overlapping oligonucleotide (~45 bp) that span the coding region and hybridizing them to tissue microarrays containing hundreds of sections (including xenografts) described in the methods and materials. The signal was quantified using a Fuji phosphoimager and scanner and Bos software. The quantification of each of these there methods is plotted above the images for each of the nine xenografts. The absolute values are normalized to three of the primary tumors with the lowest Northern hybridization levels. For each of the xenograft tumors, the S100P protein expression is shown by IHC staining is shown below the graph (FIG 4).

FIG 4 shows that in at least the xenograft samples there is good concordance between Northern hybridization, cDNA microarray, and mRNA *in situ* on tissue microarray quantitation of S100P transcript levels. These mostly but not always correlate with immunohistochemical staining. In at least a few cases, higher protein expression was observed with moderate levels of mRNA, indicating possible post-transcriptional regulation.

In situ mRNA hybridization was also used to quantitatively measure transcript levels on tissue microarray sections. Immunohistochemical analysis of S100P protein expression in 440 human prostate cancer specimens at various stages of progression is shown in FIG 5. An S100P-specific antibody was used to stain prostate tissue sections on a tissue microarray. The staining intensity was scored by two pathologists, using a scale of from 0 to 4. The results in FIG 5 show the percentage of cancers at each stage of prostate cancer progression that had strong staining (score of 3 or 4). FIG 5 shows that the high expression of S100P protein is associated with progression in clinical prostate cancers, with increasing expression in refractory and metastatic disease.

FKBP5

Translation of the observations on FKBP5 to clinical specimens is of interest because of this protein is associated with therapeutic response, and is over-expressed in recurrent tumors. Until now, it was thought that FKBP5 was only expressed in T-cells, and that it would make a good drug target for specific immunosuppression through the inhibition of glucocorticoid receptor transcriptional activation. Using prostate cancer progression tissue microarrays, FKBP5 was found to be expressed specifically in secretory cells of the normal prostate and in prostate cancer cells, but not in supporting stromal cells. Analysis of FKBP5 protein expression by IHC on the same prostate cancer tissue microarray as discussed above indicated that FKBP5 is expressed in the majority of prostate cancers,

but an association with progression was not observed. Many of the primary and early lesions had common expression of this protein, thereby indicating that FKBP5 would not make a good biomarker for prostate cancer progression or the development of hormone refractory or metastatic disease. However, FKBP5 down-regulation does appear to be associated with therapeutic response, making it a candidate for therapeutic targeting in a large percentage of clinical tumors.

LMO4 and *CRYM*

LMO4 and *CRYM* genes were substantially down-regulated in the CWR22R tumors relative to primary CWR22, for mRNA ISH studies. In both cases, mRNA ISH on TMAs validated the relative expression levels seen by cDNA microarrays in the CWR22 xenograft specimens. This analysis revealed a lower level of *LMO4* and *CRYM* expression in 17 recurrent CWR22R xenografts ($p<0.001$) as compared to 19 primary CWR22 xenografts. In addition to permitting us to validate our observations, the xenografts on the tissue microarrays were also used to compare the measurement of mRNA by cDNA microarray and mRNA ISH on a tissue microarray. As an example, there is a high correlation ($r=0.96$, $n=16$) between the levels of LMO4 mRNA measured by mRNA ISH on a tissue microarray and data from cDNA microarrays.

A significant decrease ($p<0.001$) of mRNA levels was observed for both *LMO4* and *CRYM* during tumor progression in cancer patients by mRNA ISH on the TMA. The mean intensity of actin mRNA was used as a negative control in the mRNA ISH. Comparison of mRNA ISH levels between primary and hormone refractory tumors on the same array revealed no significant differences between the two groups ($P = 0.927$).

Since antibodies are often not available for gene products discovered from cDNA microarray surveys, it remains essential to detect these transcripts on tissue microarrays using mRNA ISH. We validated here mRNA ISH-based detection of transcripts by inserting into the TMAs specimens that were originally used in the cDNA microarray analyses. There was an excellent correlation between mRNA ISH and cDNA microarray results, indicating that this method can be used to accurately measure mRNA levels in samples on a tissue microarray format. mRNA ISH was performed with several radioactively labeled oligonucleotide probes for different regions of the target genes. The use of short probes to different regions of the genes made it possible to obtain a signal even from degraded mRNAs that inevitably exist in clinical specimens. CRYM and LMO4 were down-regulated in clinical specimens from hormone-refractory tumors, which is in line with the cDNA microarray results in the CWR22 xenograft model system.

LMO4 is a member of the LIM-only (LMO) subfamily of LIM domain-containing transcription factors that is expressed during embryonic development (Kenny *et al.*, *Proc. Natl. Acad. Sci.* 95:11257-11262, 1998) and Crystallin mu (*CRYM*) codes for a thyroid hormone binding protein (Kim *et al.*, *Proc. Natl. Acad. Sci.* 89:9292-9296, 1992; Aoki *et al.*, *J. Invest. Dermatol.* 115:402-405, 2000). Both had transcript levels that were negatively associated with clinical progression. A role in prostate cancer progression has previously not been reported for either of these genes. It is believed

that the observations presented herein indicate that perturbation of these genes has a functional role in clinical prostate cancer progression and pathogenesis.

This example illustrates tissue microarray technology validation of the *in vivo* involvement of four new prostate cancer related genes. Alterations in S100P, FKBP5, CRYM and LMO4 genes are not only involved in the acquisition of androgen-independent growth and failure of therapy in prostate cancer xenografts but also with the progression of cancer in patients.

EXAMPLE 5

Targeting Candidate Genes with Known Drugs

This example demonstrates the clinical effectiveness of selecting drug targets and genetic markers, indeed entire metabolic pathways, using the herein-disclosed HRPC-related genes. Several drugs were identified based on their known interaction with one or more of the HRPC-related genes or implicated pathways, and the activities of these drugs in controlling prostate cancer cell growth was examined.

Cell viability and Drug Treatment

Exponentially growing LNCaP or CWR22R cells were trypsinized and plated at 0.5×10^5 cell/ml or 1×10^5 cell/ml respectively in 96-well culture plates. After 24 hours, cells were treated for 72 hours with serial twofold dilutions of compound. DMSO was added to the control wells. Cell viability was measured by the WST-8 assay (Dojindo Molecular Technologies Inc.). The WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2H tetrazolium, monosodium salt] assay is based on the conversion of the tetrazolium salt WST-8 to highly water soluble formazan by viable cells (Tominaga *et al.*, *Anal. Commun.* 36, 47-50, 1999). The WST-8 reagent solution was added to each well. After incubation for three hours at 37 °C, the absorbance was measured at 450 nm with a reference wavelength at 630 nm. The experiments were performed in triplicate. The data are representative of three separate experiment.

MS-275 and TSA

A literature search, coupled with a search of previous drug treatment data, was used to identify known compounds that could be used to target one or more of the 604 genes that changed at least two-fold following therapy response (FIG 1 and Table 1), or one or more of the 251 genes that were differentially expressed between primary and recurrent tumors (Table 6).

ID3 (clone: 756405) has recently been shown to be required for angiogenesis (Lyden *et al.*, *Nature* 401:670-677, 1999). The inventors have also observed a decreased in thrombospondin (clone: 810512) (an angiogenesis inhibitor) during prostate cancer progression, suggesting that the expression of these two genes is changed in opposite directions in recurrence to achieve the same biological outcome, increased angiogenesis. Currently, there are no known inhibitors of ID3 (clone:

756405), but the inventors have observed in an independent set of cDNA microarray experiments that TSA, induced thrombospondin (clone: 810512) by as much as 8.6 fold in PC3M cells *in vitro*.

TSA works by an unknown mechanism, possibly by histone deacetylase inhibition resulting in altering transcription of a large number of genes. TSA treated PC3M cells revealed targets that were similarly affected in the direction of the growth arrested xenografts. Both drugs reduced kallikrein 3 (prostate specific antigen) (clone 824568) by two-fold, possibly reflecting an inhibition of AR-dependent transcriptional activation. Histone acetyltransferase 1 (clone: 745560) and acetyl-Coenzyme A acyltransferase (clone: 27848) are both decreased by about two-fold after castration, indicating that histone deacetylase inhibition might mimic this effect (growth suppression).

Cyclin D1 (clone 841641) mRNA levels dropped to about 50 % only slightly after castration but the CWR22R recurrent tumors overexpressed it relative to the primary. Similarly chromosome condensation 1 (clone 724615) was 2.8 times higher (pooled experiment) in recurrent tumors. Both Cyclin D1(clone 841641) and chromosome condensation 1 (clone 724615) were repressed by about three-fold by TSA treatment. The recurrent to primary ratio for protease inhibitor 12 (neuroserpin) (clone 564621) was 0.27, but TSA induced it by 8.67 fold.

Gene expression changes in response to treatment with these two drugs indicated that they might restore the expression of several genes that are associated with therapy resistance in CWR22R xenografts. TSA effectively inhibited growth of CWR22R, as indicated in FIG 3D. It is not known which of the above mentioned targets were affected, or by which mechanisms these two drugs caused growth arrest. It is possible that these drugs had a more global gene expression effect, which simultaneously restored multiple androgen responsive genes that are required for growth in the recurrent tumors.

Rapamycin and FK506

Sirolimus (Rapamycin) and Tacrolimus (FK506) are bacterial macrolides that are produced by fungi to suppress the growth of competing organisms. These drugs are immunosuppressants used extensively to prevent organ rejection. Although the two drugs are very similar both in structure and in their cellular targets, known as immunophilins (also called FKBP for FK506 binding proteins), the mechanism by which they cause immunosuppression is different. FK506 binds to immunophilins and the complex inhibits calcineurin in T-cells. In contrast, rapamycin-immunophilin complex inhibits signaling of the S6-kinase (clone: 204148, which also responds to castration) causing cell cycle arrest in T-cells. In addition, there are "macro" immunophilins that have been found to interact with steroid receptors, which may work though yet another mechanism to inhibit growth when complexed with these drugs.

Several drug targets identified in this study are involved in immunophilin pathways, suggesting that either FK506 or rapamycin may cause a growth inhibition of hormone refractory prostate cancer. The first such candidate is a macroimmunophilin called FKBP5 (clone: 416833), one of the most strongly repressed genes in primary prostate CWR22 tumors after castration (FIG 2). The

expression of FKBP5 (clone: 416833) is restored in hormone refractory CWR22R prostate cancer. In some tumors, FKBP5 mRNA expression (determined using cDNA microarray and RT-PCR quantitation) is restored to levels higher than found in the primary tumors. The availability of FKBP5 as a drug target was also confirmed using tissue microarray analysis. It is not clear if FKBP5 is required for the proliferation of CWR22R cells, but the expression of the FKBP5 transcript is associated with the proliferation phenotype. FKBP5 is a large protein that associates with steroid receptors, such as the glucocorticoid receptor, through binding to HSP90. It is also possible that FKBP5 interacts with the AR.

Cyclin D mRNA was 2.5-fold higher in a pool of four recurrent tumors compared to a pool of four recurrent tumors. Rapamycin has been shown to target and down-regulate cyclin D protein at both a transcriptional and post-transcriptional level (Hashemolhosseini *et al.*, *J. Biol. Chem.* 273:14424-14429, 1998). Also, p27 had increased after castration by about 2-fold by day 8, and then went back down in the recurrent tumor. Rapamycin can increase p27 levels, making it a candidate for reversing the decrease seen in the recurrent CWR22R. These rapamycin effects on both cyclin D and p27 may be direct, but also may be mediated by inhibition of the phosphotidylinositol 3-kinase/PTEN/AKT/FRAP pathway. Several gene expression changes have been identified herein that could converge to activate this pathway in recurrent tumors, further suggesting that this is a pathway necessary for androgen independent growth. For example, an increased was observed in expression of CDP-diacylglycerol synthase 1 (levels up to 2.77-fold higher in recurrent tumors). CDP-diacylglycerol synthase 1 is a rate limiting enzyme in phosphotidylinositol 3 (PI3) production that has been shown to increase the amplitude and duration of PI3 signaling when overexpressed in model systems. PTEN, which is an inhibitor of this pathway, is increased during regression and re-expressed in the recurrent tumors further illustrating the importance of this pathway for proliferation of recurrent tumors.

It has also been shown that rapamycin inhibits the translation of ornithine decarboxylase (ODC) transcripts by about 50% in epithelial cells. In this study, ODC was repressed (3.8 fold) during CWR22 regression, but then re-expressed in the recurrent CWR22R (FIG 2). Interestingly, FK506 has no effect on ODC transcript levels. Both ODC and cyclin D are important stimulators of proliferation, indicating that rapamycin can be used to target these molecules and cause growth arrest in androgen independent CWR22R cells.

Rapamycin effectively arrested the CWR22R cells *in vitro,* however a complete inhibition was not accomplished at the highest concentration of FK506 (10μM) (FIG 3D). It is believed that the interaction of rapamycin with FKBP5 and its other cellular receptor immunophilins blocks a pathway necessary for growth, while the interaction of FK506 and FKBP5 does not. It is difficult to predict the mechanism by which these drugs exert an effect on a cell, because they bind multiple cellular targets. In this case, several putative cellular targets are known for these two macrolide drugs and at least one, FKBP5, was both associated with the HRPC phenotype, and available in the relevant cells

(FKBP5 protein is expressed in most clinical recurrent tumors). More specific inhibitors of FKBP5 activity can be used to elucidate the role FKBP5 plays in the growth of hormone refractory tumors.

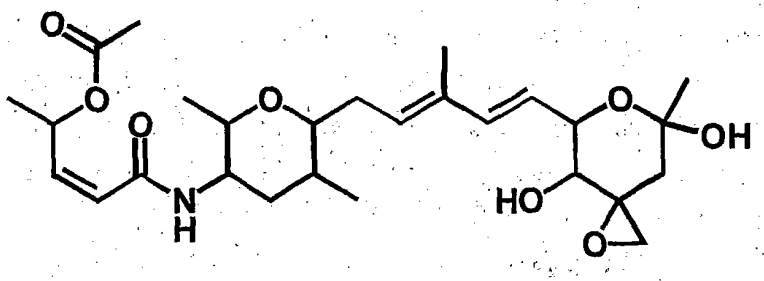
EXAMPLE 6

Pharmacogenomics Analysis

This example illustrates the involvement of gene targets in pharmacological response to various emerging therapies.

Xenografts and Cell lines: Fresh frozen tissue from CWR22 human prostate cancer xenografts (Pretlow *et al.*, *J. Natl. Cancer Inst.* 85:394-398, 199)) was obtained from thirteen different mice at different stages of hormonal therapy and tumor progression (four primary untreated CWR22, five CWR22 therapy time points after 0.5 days, 2 days, 4 days, 8 days and 16 days after castration, and four independent hormone-refractory CWR22R strains). LNCaP (ATCC) and CWR22R (kindly provided by Dr. Jim Jacobberger's Laboratory at Case Western University) cell lines were cultured in RPMI1640 10% fetal bovine serum (Life Technologies Rockville, MD) at 37 °C and 5% CO_2. mRNA was extracted with the FastTrack 2.0 Kit (Invitrogen Corporation; Carlsbad, California).

Drug Treatment and Cell viability: Exponentially growing LNCaP or CWR22R cells were trypsinized and plated at 0.5 x 10^5 cells/ml or 1 x 10^5 cells/ml respectively in 96-well culture plates. After 24 hours, cells were treated for 72 hours with serial two-fold dilutions of either FK-506 (Tacrolimus, Calbiochem Inc., San Diego, California), Rapamycin (Sirolimus) (Sigma Chemical co. St. Louis, Missouri), FR901464 (Fujisawa Pharmaceutical Co., Ltd., Ibaraki, Japan), Trichostatin A - TSA (a histone deacetylase inhibitor; Sigma Chemical co. St. Louis, Missouri) or DMSO as a control. The structure of FR901464 is as follows:



Cell viability was measured (triplicate experiments) by the WST-8 assay (Dojindo Molecular Technologies Inc., Gaithersburg, Maryland). CWR22R cells were treated with various drugs at effective doses for 1, 3, 9 and 24 hours followed by mRNA isolated for cDNA microarray experiments.

Analysis of mRNA expression by cDNA Microarrays: Custom cDNA microarrays were constructed consisting of 6605 to 8000 elements (sequence verified clones from Research Genetics, Huntsville, Alabama), representing different (non-redundant) transcripts including 4032 to 7700 known (named) genes (Mousses *et al.* in *Functional Genomics*, (eds. Livesey & Hunt) 113-137,